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The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the *Saccharomyces cerevisiae CTT1* gene

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The HOG signal pathway of the yeast *Saccharomyces cerevisiae* is defined by the *PBS2* and *HOG1* genes encoding members of the MAP kinase kinase and of the MAP kinase family, respectively. Mutations in this pathway (deletions of *PBS2* or *HOG1*, or point mutations in *HOG1*) almost completely abolish the induction of transcription by osmotic stress that is mediated by stress response elements (STREs). We have demonstrated previously that STREs also mediate induction of transcription by heat shock, nitrogen starvation and oxidative stress. This study shows that they are also activated by low external pH, sorbate, benzoate or ethanol stress. Induction by these other stress signals appears to be HOG pathway independent. *HOG1*-dependent osmotic induction of transcription of the *CTT1* gene encoding the cytosolic catalase T occurs in the presence of a protein synthesis inhibitor and can be detected rapidly after an increase of tyrosine phosphorylation of Hog1p triggered by high osmolarity. Consistent with a role of STREs in the induction of stress resistance, a number of other stress protein genes (e.g. *HSP104*) are regulated like *CTT1*. Furthermore, catalase T was shown to be important for viability under severe osmotic stress, and heat shock was demonstrated to provide cross-protection against osmotic stress.

Key words: HOG pathway/MAP kinase/*Saccharomyces cerevisiae*/STRE/stress

Introduction

Cells respond and adapt to environmental stress by immediate or early responses that do not require the synthesis of new proteins but involve the modulation of pre-existing proteins to produce changes e.g. in metabolism and membrane transport. Such changes may require the activation of signal transduction pathways by stress signals. As part of the immediate response, such signalling pathways may also induce changes in gene expression which provide the basis for the delayed or late responses. The combination of early and late responses enables cells to adapt and to resume growth under mildly stressful conditions and to survive under more severe conditions (induced stress resistance). The coupling between early and late responses that is mediated by signal protein-regulated changes in stress gene transcription is not yet well understood.

Stress by increases in external osmolarity reduces growth and viability of yeast cells due to a range of effects that include loss of an osmotic gradient across the plasma membrane and, under extreme conditions, cell shrinkage (Mager and Varela, 1993). Yeast cells adapt to the addition of high concentrations of NaCl or other solutes to their medium by accumulating glycerol, thus increasing internal osmolarity (Blomberg and Adler, 1989). Glycerol accumulation results in part from the increased activity of glycerol-3-phosphate dehydrogenase encoded by the *GPD1* gene (Larsson *et al.*, 1993). In addition to *GPD1*, osmotic stress-stimulated glycerol accumulation requires two other genes, *HOG1* and *PBS2* (Boguslawski, 1992; Brewster *et al.*, 1993). *HOG1* encodes a member of the MAP (mitogen-activated protein) kinase family, *PBS2* codes for a representative of the MAP kinase kinase family. The putative protein kinases encoded by these genes constitute an osmosensing signal transduction pathway called the HOG (high osmolarity glycerol response) pathway. Immunoblot analysis using an anti-phosphotyrosine antibody demonstrated that Hog1p is phosphorylated on tyrosine residues in cells exposed to an increase in osmolarity. This phosphorylation is induced rapidly (<1 min), reaches a maximum at 300 mM NaCl and requires *PBS2*.

While the yeast *Saccharomyces cerevisiae* exhibits characteristic responses to a variety of other stress conditions, the mechanisms of transduction of these stress signals are largely unknown. Among stress conditions, heat shock has been studied most extensively (for reviews see e.g. Craig, 1992; Mager and Moradas Ferreira, 1993). An increase in temperature induces the accumulation of a small set of proteins, at least partly by inducing transcription of heat shock genes. Some of the heat shock-induced proteins (hsps) facilitate growth at higher temperatures and lead to thermotolerance, the ability to survive at higher temperatures (Piper, 1993). Elevated temperatures induce transcription via at least two different types of upstream activating sequence (UAS) elements. Heat shock elements (HSEs) specifically bind heat shock transcription factor (HSF) (Sorger, 1991; Lis and Wu, 1992) and are activated not only by heat shock, but also by some other stress signals (Burdon, 1986). Another type of UAS element that mediates heat shock induction of transcription independent of HSF has more recently been identified in the promoters of the *CTT1* (catalase T) and the *DDR2* (also induced by DNA damage) genes of *S.cerevisiae* (Wieser *et al.*, 1991; Kobayashi and McEntee, 1993). A 13 bp DNA sequence of *CTT1* unrelated to HSEs has been demonstrated to be sufficient for the activation of a reporter gene by heat shock (Marchler *et al.*, 1993). In

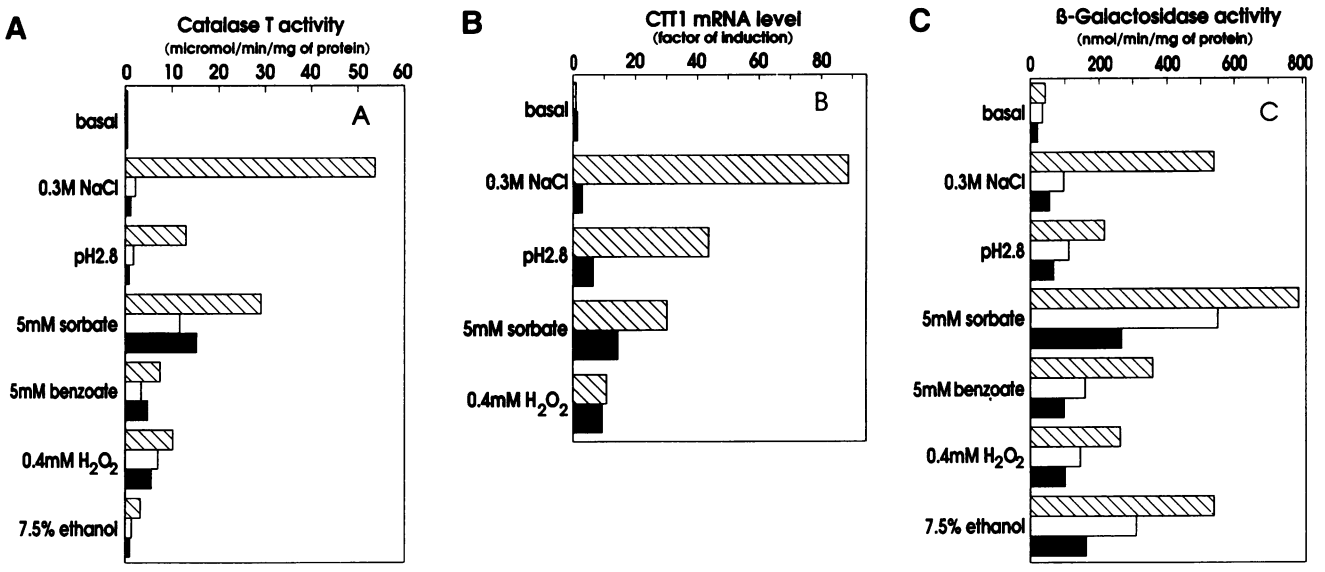

Fig. 1. Effect of disruption of *PBS2* and *HOG1* genes on stress induction of catalase T activity (**A**), *CTT1* mRNA (**B**) and of an STRE-driven *LEU2–lacZ* reporter gene (**C**). Logarithmic cultures of strain GG18 (hatched bars), GG18pbs2 (open bars) and GG18hog1 (closed bars) grown on YPD were subjected to various stress conditions. Data presented are the mean of at least three independent experiments. Northern blots were probed with *CTT1* and *ACT1* fragments. Quantitative evaluation of band intensities was carried out using a PhosphorImager (Molecular Dynamics). *CTT1* mRNA levels were corrected using *ACT1* mRNA as a non-regulated internal control and are presented relative to basal level (unstressed wild type control).

contrast to HSEs, this element is under negative control by protein kinase A and is activated by nitrogen starvation (Marchler *et al.*, 1993) or in stationary phase cells (C. Schüller, unpublished results). Furthermore, this DNA element, which has been called stress response element (STRE), mediates activation of transcription by hydrogen peroxide and increases in osmolarity.

Because of their activation by various types of stress, both HSEs and STREs may be involved in the induction of stress resistance of yeast and of other organisms. If they do have this type of function, products of genes induced via such elements should possess protective functions under stress conditions. While it has been generally difficult to prove such a function in the case of a number of yeast hsps, it has been demonstrated most clearly for the product of the *HSP104* gene (Sanchez *et al.*, 1992). A positive effect on thermotolerance of yeast has also been shown for catalase T (Wieser *et al.*, 1991). If HSEs or STREs are involved in the induction of stress resistance, their induction of transcription by one type of signal should provide protection of cells against other stresses activating the same element (cross-protection). Cross-protection by nutrient deprivation and heat shock (Plesset *et al.*, 1987) are consistent with a role of STREs in the induction of stress resistance. However, cross-protection by some other factors activating STREs has not yet been demonstrated.

Like heat shock, osmotic stress induces the synthesis of a small set of proteins. The profile of proteins induced by the two types of stress overlaps, but appears largely distinct (Varela *et al.*, 1992). One of the proteins induced by both types of stress is catalase T, and the STRE of the *CTT1* gene has been shown to be sufficient for this response (Marchler *et al.*, 1993). Since the signalling proteins necessary for this delayed stress response are unknown and since signal pathways containing MAP kinases are known to regulate transcription (Neiman, 1993; Errede and Levin, 1993) we investigated whether the HOG pathway mediates osmotic regulation of *CTT1* transcription and whether STRE is a target of signals mediated by this pathway. The results obtained in our investigation show that STRE-mediated induction of *CTT1* expression is defective in strains lacking functional *HOG1* or *PBS2* and that the induction of expression of some other yeast genes, among them *HSP104*, is also redued in *hog1* null mutants. Our results also provide evidence for an important role of catalase T in protection against osmotic stress and for cross-protection against this stress by mild heat stress. These observations are consistent with an important role of STREs in the induction of stress resistance.

Results

The HOG pathway regulates osmotic induction of CTT1 via STRE

Since mutations of *HOG1* or *PBS2* render cells sensitive to high osmolarity (Boguslawski, 1992; Brewster *et al.*, 1993), it seemed plausible that the HOG pathway regulates osmotic induction of transcription. Therefore, we tested whether mutations in this pathway cause alterations in gene expression driven by STREs. *HOG1* and *PBS2* were deleted in strain GG18 by gene disruption. In accordance with published data, the resulting mutants were osmosensitive. Catalase T activity, CTT1 mRNA levels and STRE-driven β-galactosidase (a single copy chromsosomal integrant of an STRE–*LEU2–lacZ* reporter gene construct; pCTT1–18 7x; Marchler *et al.*, 1993) expression were assayed in stressed wild type and mutant cells (Figure 1). Catalase T activity (Figure 1A) in cells treated with 0.3 M NaCl was dramatically reduced in GG18hog1 and in GG18pbs2 compared with the wild type strain. A similar effect was observed when we assayed *CTT1* mRNA (Figure 1B; only GG18hog1 assayed) and in the case

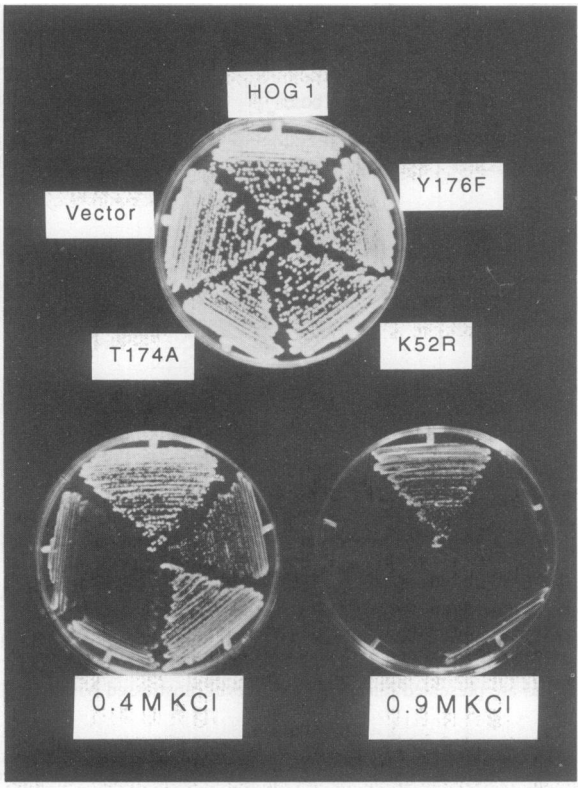

Fig. 2. *HOG1* mutant genes will not support growth at high osmolarity. To evaluate the ability of each point mutant to rescue cell growth under osmotically stressed conditions, single copy vector pRS316 or derivatives carrying *HOG1*, *hog1*-K52R, *hog1*-T174A and *hog1*-Y176F genes, respectively, were transformed into strain JBY13 (*hog1*). Growth was evaluated by streaking cells on YPD and YPD containing 0.4 M or 0.9 M KCl, respectively (NaCl was also tested with similar results). Plates were incubated for 36 h at 30°C.

Table I. Catalase T induction by osmotic stress requires a functional *HOG1* gene

Strain JBY13 (*hog1*) transformed with plasmid	Catalase T activity (μmol/min/mg protein)[a]	
	Control (1 h)	0.3 M NaCl (1 h)
pRS316 (vector)	0.2 ± 0.2	0.9 ± 0.0
pHOG1	1.7 ± 0.1	18.6 ± 1.6
phog1-Y176F	0.8 ± 0.5	1.0 ± 1.0
phog1-T174A	0.4 ± 0.3	0.7 ± 0.3
phog1-K52R	0.4 ± 0.2	2.3 ± 0.1

[a]Mean ± SD for three independent cultures

of STRE-driven β-galactosidase production (Figure 1C). Induction of transcription by 0.4 M sorbitol was affected by the mutations to a similar extent (data not shown).

In addition to the *pbs2* and *hog1* deletion mutants, point mutants affecting activation (T174A, Y176F) or ATP binding (K52R) of the Hog1p protein kinase were also tested. As demonstrated in Figure 2, cells expressing any of the mutant Hog1p kinases on a single copy plasmid in a *hog1* deletion background (JBY13) were osmotically sensitive. Consistent with this observation, catalase T is not inducible in either *hog1*-Y176F or *hog1*-T174A and is induced only to a low level in the *hog1*-K52R mutant (Table I). As expected, we observed no tyrosine phosphorylation in the case of the *hog1*-Y176F mutant (Figure 3), whereas basal levels of *hog1*-K52R and particularly *hog1*-T174A tyrosine phosphorylation were higher than in the case of wild type Hog1p, but were still inducible in cells exposed to osmotic shock. The data obtained demonstrate that all genetic defects affecting activity or levels of Hog1p cause corresponding effects on STRE-driven expression.

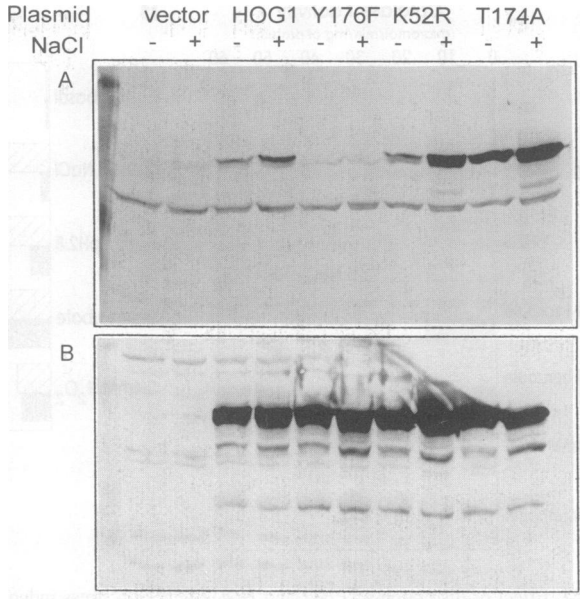

Fig. 3. Phosphotyrosine analysis of *HOG1* point mutants. Immunoblot analysis was performed on strain JBY13 (*hog1*) transformed with multicopy plasmid pRS426 or with pRS426 carrying *HOG1*, *hog1*-K52R, *hog1*-T174A or *hog1*-Y176F inserts, respectively. Cells were grown overnight in selective medium, diluted in YPD for 3–6 h to allow cells to reach logarithmic phase, and divided into aliquots. Total cellular protein was isolated immediately or following a 10 min exposure to 0.4 M NaCl. Duplicate Western blots were performed (40 μg protein loaded per lane). Primary antibodies were antiphosphotyrosine (**A**) or affinity purified RC6 serum specific for Hog1p (**B**).

STRE sequences are activated by a broad variety of stress signals, but HOG pathway control appears limited to osmotic stress

Previous work has shown that, in addition to osmotic stress, STREs are activated by heat shock, nitrogen starvation and oxidative stress (Marchler *et al.*, 1993). In the present study we found sorbate (5 mM), benzoate (5 mM), ethanol (7.5% v/v) and low external pH (pH 2.8) to be effective and rapid (30–60 min) inducers of *CTT1* expression (Figure 1). Both the chromosomal wild type *CTT1* gene and the STRE−*LEU2*−*lacZ* reporter gene construct are induced in logarithmic phase cells exposed to various stress conditions. No induction was observed by these conditions when the *LEU2*−*lacZ* integration vector pLS9 was used (data not shown).

As also demonstrated in Figure 1, *pbs2* and *hog1* mutations had a negative effect on the activation of STREs by all stress factors tested (Figure 1C). Furthermore, cells from *hog1* strains had reduced levels of β-galactosidase and catalase T activity under heat shock, growth on ethanol-containing medium and starvation for nitrogen (data not shown). However, factors of induction compared with basal levels are significantly (>50%) reduced by the mutations only in the case of osmotic stress. This indicates that the HOG pathway is not directly involved in the

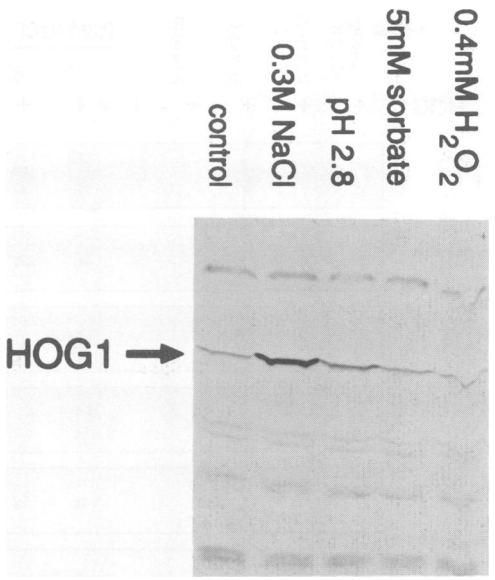

Fig. 4. Hog1p tyrosine phosphorylation is induced only by osmotic stress. Immunoblot analysis was performed using strain JBY13 (*hog1*) transformed with multicopy vector pRS426 (Sikorski and Hieter, 1989) carrying a wild type *HOG1* gene. Logarithmic cells were exposed to various stress conditions. Total cellular protein was prepared after exposure to stress for 10 min. Primary antiphosphotyrosine antibodies were used to visualize tyrosine-phosphorylated Hog1p.

transmission of these other stress signals. To obtain further evidence, tyrosine phosphorylation of Hog1p was studied in cells exposed to various types of stress. This phosphorylation has been correlated with activation of the HOG pathway in cells exposed to mild osmotic stress (0.4 M NaCl) (Brewster *et al.*, 1993). Tyrosine phosphorylation of Hog1p expressed on a multicopy plasmid is detectable with antiphosphotyrosine antibodies. The result of an experiment analyzing the phosphorylation status of Hog1p from cells exposed to inducing levels of NaCl, sorbate, H_2O_2 and to low external pH are shown in Figure 4. As observed previously, Hog1p tyrosine phosphorylation increased upon treatment with 0.4 M NaCl, but no significant increase was observed under other stress conditions. The equal intensity of non-specific bands in different lanes of the blot shows that protein loading in different lanes was fairly constant. When a separate blot was treated with an anti-Hog1p antibody, a constant signal was obtained (not shown). This demonstrates that levels of Hog1p were not significantly altered during exposure to stresses. These observations are consistent with the conclusion that activation of STREs by the HOG pathway is limited to osmotic stress.

HOG pathway dependent induction of CTT1 is rapid and occurs in the absence of protein synthesis

To assay whether the HOG pathway effect on *CTT1* expression is an early response to osmotic stress and independent of protein synthesis, we analyzed the kinetics of induction and the effect of cycloheximide on the induction of *CTT1* mRNA. Catalase T activity is detectable as early as 12 min after treatment with 0.3 M NaCl (not shown). Consistent with this result we found that induction of *CTT1* mRNA is very rapid. It is significantly increased after 3 min (see Figure 7). Because of this time scale

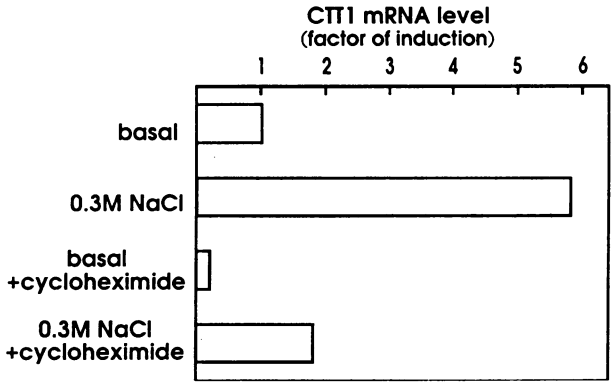

Fig. 5. Osmotic induction of CTT1 mRNA is not dependent on *de novo* protein synthesis. A logarithmic culture of strain GG18 was split, and one half was treated with cycloheximide (100 μg/ml) for 15 min, while the other half was left as untreated control. Then both cultures were split again and were incubated for 20 min with or without addition of 0.3 M NaCl. RNA was prepared from cells and was hybridized with *CTT1* and *ACT1* probes after electrophoresis and transfer to membranes. Quantitative evaluation of band intensities was carried out using a PhosphorImager. *CTT1* mRNA levels are corrected using *ACT1* mRNA as internal control and are presented relative to basal level (cells not treated with cycloheximide or NaCl).

we expected the induction of *CTT1* transcription to be independent of *de novo* protein synthesis. Logarithmic cultures were therefore exposed to 0.3 M NaCl for 20 min in the presence of cycloheximide. Simultaneously, induction of catalase activity was used as a convenient and sensitive assay for the synthesis of protein. Catalase activity was not detectable in cycloheximide-treated cultures (not shown). Northern analysis (Figure 5) demonstrated that *CTT1* mRNA levels were reduced in the presence of cycloheximide under basal as well as under inducing conditions, but that significant induction occurred in the presence of the protein synthesis inhibitor. From these data we suggest that the activation of STRE-dependent transcription by osmotic stress is a direct effect.

Relationship of HOG and protein kinase A pathways in the control of STRE activity

Protein kinase A plays an important role as a negative regulator of *CTT1* via STREs (Belazzi *et al.*, 1991; Marchler *et al.*, 1993). Since published evidence (Boguslawski, 1992) suggests that *PBS2* (and *HOG1*) might act downstream of the RAS-cAMP-protein kinase A pathway, we analyzed the osmotic induction of an STRE−*LEU2*−*lacZ* reporter gene in mutants affecting protein kinase A activity. The *RAS2* gene of *S.cerevisiae* encodes an activator of cAMP-dependent protein kinase. Therefore, *ras2* mutants have reduced protein kinase A activity. *BCY1* codes for the regulatory subunit of protein kinase A. Deletion of *BCY1* results in high, cAMP-independent protein kinase A activity. As expected from previous experiments, the *ras2* mutant tested exhibits an enhanced β-galactosidase level in the absence of osmotic stress. However, expression induced by 0.3 M NaCl is not significantly affected (Table II). The *bcy1* mutation reduces expression in both osmotically stressed and control cells, but does not significantly affect the factor of induction by osmotic stress or by other types of stress (not shown). Consistent with protein kinase A and HOG pathways acting in parallel, the *HOG1*-independent expres-

Table II. Effects of activity of the protein kinase A pathway on HOG pathway mediated induction of STRE−*LEU2*−*lacZ* expression

Strain	β-Galactosidase activity (nmol/min/mg protein)[a]	
	Control (1 h)	0.3 M NaCl (1 h)
GG18	44.8 ± 17.3	539.0 ± 26.4
GG18hog1	21.3 ± 7.4	55.9 ± 15.4
GG18ras2	289.1 ± 38.7	628.4 ± 98.5
GG18hog1ras2	87.2 ± 35.7	113.4 ± 30.8
GG18bcy1	4.7 ± 2.0	22.0 ± 9.8
GG18hog1bcy1	3.5 ± 1.1	5.8 ± 3.1

[a]Mean ± SD for at least three independent cultures.

CTT1	-381	CAAGGGGA	-374
	-366	TAAGGGGC	-359
DDR2	-172	TAAGGGGT	-179
HSP104	-254	TAAGGGGC	-247
	-222	ACAGGGGG	-215
	-174	AAAGGGGC	-167
GAC1	-661	TCAGGGGG	-654
UBI4	-659	CCAGGGGA	-666
	-254	TCAGGGGC	-247
HSP12	-661	TCAGGGGG	-654
	-478	TTAGGGGG	-485
	-460	TTAGGGGG	-467
	-322	TAAGGGGG	-329
HSP26	-437	TTAGGGGA	-430
	-416	GCAGGGGG	-409
	-379	AGAGGGGA	-372
	-226	CCAGGGGC	-233

Fig. 6. STRE-related sequences in the upstream regions of stress-induced yeast *S.cerevisiae* genes (*CTT1*: Hartig and Ruis, 1986; *DDR2*: Kobayashi and McEntee, 1990; *HSP104*: EMBL data bank accession number M67479; *GAC1*: Francois *et al.*, 1992; *UBI4*: Özkaynak *et al.*, 1987; *HSP12*: Praekelt and Meacock, 1990; *HSP26*: Petko and Lindquist, 1986).

sion of the STRE−*LEU2*−*lacZ* gene observed in strain GG18hog1 is significantly reduced by a *bcy1* disruption mutation (GG18hog1bcy1) in the presence as well as in the absence of osmotic stress. The partial epistasis of the *hog1* over the *ras2* mutation observed in the double mutant strain GG18hog1ras2 can also more easily be reconciled with the existence of two parallel pathways than with a linear pathway involving components of the RAS-cAMP-protein kinase A and of the HOG pathway.

A set of S.cerevisiae genes is controlled in a manner characteristic of STRE-activated expression

The phenomenon of cross-protection probably involves the products of many different genes. Consistent with the hypothesis that STREs play an important role in this mechanism, STRE-like sequences are found in the promoter region of a number of yeast genes (Figure 6). To obtain evidence for STRE-mediated control of such genes, RNA from wild type (GG18) and GG18hog1 cells exposed to 0.3 M NaCl for 3, 15 and 60 min or to 5 mM sorbic acid, 0.4 mM H_2O_2 or pH 2.8 for 20 min was prepared and subjected to Northern analysis (Figure 7). Expression of *DDR2*, which encodes a DNA damage inducible gene and has been reported to be induced by heat shock in an HSF-independent manner (Kobayashi and McEntee,

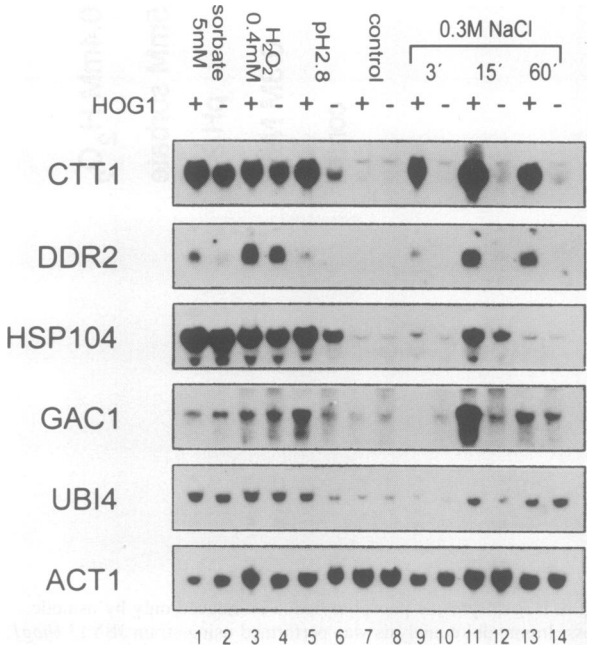

Fig. 7. Similar pattern of stress control of genes containing STRE-like sequences in their promoter. Logarithmic cultures of strain GG18 (lanes 1, 3, 5, 7, 9, 11 and 13) and GG18hog1 (lanes 2, 4, 6, 8, 10, 12 and 14) were divided into aliquots and exposed to 5 mM sorbic acid (lanes 1 and 2), 0.4 mM H_2O_2 (lanes 3 and 4) and pH 2.8 (lanes 5 and 6) for 20 min. Lanes 7 and 8 represent untreated control. Lanes 9–14, cells exposed to 0.3 M NaCl for 3 min (lanes 9 and 10), 15 min (lanes 11 and 12) and 60 min (lanes 13 and 14). RNA was prepared and probed with *CTT1*, *DDR2*, *HSP104*, *GAC1*, *UBI4* and *ACT1* fragments after electrophoresis and blotting.

1993), revealed the same general pattern of stress induction and *HOG1*-dependence as *CTT1*. The *HSP104* gene (Sanchez and Lindquist, 1990) and *GAC1*, which may encode a regulatory subunit of a protein phosphatase type 1 (Francois *et al.*, 1992), were also significantly inducible upon treatment with all stress regimes tested. Although induction of the latter two genes by osmotic stress was less *HOG1*-dependent than in the case of *CTT1* or *DDR2*, it was also reduced by the *hog1* mutation. The amount of mRNA for *UBI4*, which encodes polyubiquitin, was weakly stress inducible.

Catalase T is important for survival under extreme osmotic stress

Logarithmically growing yeast cells lose viability after exposure to medium of very high osmolarity. Incubation in 3 M NaCl kills 99% of the cells in 2 h. Pre-treatment with 0.3 M NaCl for 30 min improves survival dramatically, as 50% survive a 2 h treatment with 3 M NaCl, and 10% survive even 5 M NaCl (data not shown). Because catalase T is induced in cells exposed to 0.3 M NaCl, we looked for a physiological role of this protein under conditions of severe osmotic stress. The *CTT1* gene was deleted from strain W303-1A resulting in the derivative W303-1Actt1 and disruptants were tested for lack of catalase T activity. Logarithmic cells of W303-1A and W303-1Actt1 were exposed to 3 M NaCl in YPD medium with and without pre-treatment by mild stress. We chose 0.3 M NaCl and mild heat shock as induction conditions as both are known to induce catalase T (Wieser *et al.*, 1991; Marchler *et al.*, 1993). Survival of cells in 3 M NaCl/YPD for 8 h was

Table III. Survival of *ctt1* mutant cells after exposure to high osmolarity medium[a]

Strain	None	Pretreatment	
		0.3 M NaCl (1 h)	37°C (1 h)
W303	0.0016 ± 0.0004	20.0 ± 3.0	3.2 ± 0.3
W303ctt1	0.0008 ± 0.0002	0.7 ± 0.3	0.18 ± 0.02

[a]% viable cells (mean ± SD for three independent cultures) after treatment of YPD-grown cells with YPD−3 M NaCl for 8 h.

dramatically improved by the presence of a functional *CTT1* gene (Table III). Consistent with a role of STREs in the induction of osmotolerance, cross-protection against severe osmotic stress by prior exposure to heat shock was observed in this experiment. This type of cross-protection had not been observed by other authors (Trollmo *et al.*, 1988; Varela *et al.*, 1992).

Discussion

The data obtained in the course of this investigation demonstrate that the STRE-driven induction of transcription by osmotic stress is mediated by the HOG pathway. In yeast and in higher eukaryotes, MAP kinase-containing signal pathways regulate the transcription of genes involved in cell growth and proliferation (Pelech and Sanghera, 1992; Nishida and Gotoh, 1993). Our results show that the induction of transcription of stress response genes is also regulated by a MAP kinase pathway, extending the range of known physiological functions of this ubiquitous signalling mechanism. The induction of STRE-driven transcription via the HOG pathway in response to an increase in osmolarity has been demonstrated by our results to occur rapidly, shortly after tyrosine phosphorylation of Hog1p is detectable (Brewster *et al.*, 1993). Together with the observation that induction occurs in the presence of cycloheximide this indicates that activation of transcription occurs by post-translational modification, most likely phosphorylation, of a factor involved in signalling of increases in osmolarity. By analogy to the *S.cerevisiae* pheromone signalling pathway (Errede and Levin, 1993) and to the human JNK1 protein kinase, which is stimulated by UV light and phosphorylates the activation domain of c-Jun (Dérijard *et al.*, 1994), the target of this post-translational modification could be a transcription factor specifically binding to STREs.

In addition to the HOG pathway, two other MAP kinase pathways necessary for mating pheromone signalling and for cell wall construction or proliferation respectively, have been characterized in *S.cerevisiae* (Errede and Levin, 1993). No evidence for any contribution of these pathways to the activation of STREs has been obtained in an attempt to obtain evidence for cross-talk between structurally related signalling systems (C.Schüller, unpublished results). Concerning the specific function of the HOG pathway itself we have demonstrated that it is only involved in the activation of STREs by osmotic stress and is not necessary for the transmission of other stress signals. Other yet unidentified signal pathways have to be postulated therefore, particularly since the available data (Belazzi *et al.*, 1991; Marchler *et al.*, 1993; this investigation) do not provide any convincing evidence for a direct involvement of the protein kinase A pathway in stress signalling. It has been demonstrated that this pathway provides a positive signal activating the *S.cerevisiae* mitotic cycle (Hubler *et al.*, 1993). It is tempting to speculate that negative control of STRE-driven expression by protein kinase A provides a link between stress control and growth control. Under conditions which are generally favourable for cell division, it is selectively advantageous for a unicellular organism like yeast to direct its energy toward cell division and increasing the population size. The synthesis of stress proteins would be of minor importance under such conditions. On the other hand, lack of a signal mediated by protein kinase A slows down or stops cell division and simultaneously appears necessary for the induction of stress proteins by proper signals. Under such conditions, the protection of remaining cells by an increase in stress resistance would be of primary importance for the survival of the cell population.

Is the induction of transcription mediated by STREs an important component of the cellular system contributing to induced stress resistance? The following types of evidence are consistent with a positive answer to this question: (i) STREs are activated by a broad spectrum of stress conditions; (ii) available evidence indicates that they function in the control of transcription of a number of stress protein genes; (iii) the products of some of these genes have been shown to contribute to induced stress resistance and (iv) stress conditions activating STREs also provide cross-protection against other stress factors.

Not only STREs, but also canonical HSEs are activated by a variety of stress conditions. However, in contrast to STREs, HSEs do not respond to some conditions contributing to the induction of general stress resistance (e.g. stationary phase conditions or low protein kinase A activity) in a manner consistent with their essential role in the induction of stress resistance. As discussed earlier in more detail (Marchler *et al.*, 1993), such observations do certainly not exclude a function of HSEs in stress protection, but demonstrate that other mechanisms must exist. STRE appears to be a crucial component of such a mechanism.

While the majority of the experiments described in this paper have concentrated on the expression of the *CTT1* gene and of a reporter construct activated by an STRE derived from the upstream region of its promoter we have also made an effort to obtain information concerning the presence of STREs in other yeast genes. Data described in this paper or published previously by others demonstrate that in addition to *CTT1*, the genes *DDR2*, *HSP104*, *UBI4*, *GAC1*, *HSP12* and *HSP26* (Varela *et al.*, 1992) and *GPD1* (Blomberg and Adler, 1989) are activated by an increase in osmolarity. At least most of these osmotically regulated genes contain STRE-related sequences in the upstream regions of their promoters (Figure 6). The osmotically-induced genes we have tested in the course of this investigation show partial *HOG1* dependence of induction in response to osmotic stress and a general pattern of induction which is at least qualitatively similar to that observed for *CTT1* and for the STRE−*LEU2*−*lacZ* reporter gene. It is therefore likely that at least some of these genes contain functional STREs. The *HAL1* gene (Gaxiola *et al.*, 1992) may be an interesting exception to this rule. It has been described to be induced by 1 M

NaCl, but not by heat shock. This would be inconsistent with induction via an STRE. Furthermore, we have tested salt induction of *HAL1* by 0.3 M NaCl and the effect of a *hog1* deletion on its expression. No induction by 0.3 M salt and no *hog1* effect was detected (data not shown). It appears very likely therefore that salt induction of *HAL1* expression is mediated by a different mechanism.

The findings that the *S.cerevisiae* HSP104 (Sanchez *et al.*, 1992) and catalase T proteins (Wieser *et al.*, 1991; and this paper) do have protective functions under severe stress are also consistent with an important role of STREs in stress protection. It has to be emphasized that in both cases the mechanistic basis of this protection is presently not understood. Furthermore, although both catalase T and HSP104 have been demonstrated to make impressive contributions to stress resistance of yeast cells under special conditions, it seems clear that the response of yeast to mild stress must be much more pleiotropic and should also involve other factors (e.g. other heat shock proteins or low molecular weight compounds like trehalose).

As indicated above, any mechanism triggered by multiple stress signals and providing protection against several types of stress should cause cross-protection. Therefore, the potential relevance of STREs for the induction of stress resistance could be judged by testing whether all the signals triggering STRE-dependent transcription do provide cross-protection. While the evidence currently available is incomplete it does suggest a functional importance of STREs. Some of this evidence has been discussed recently in three reviews (Mager and Moradas Ferreira, 1993; Mager and Varela, 1993; Piper, 1993). In the present context, only a few examples should suffice to illustrate the situation. It has long been known that starvation for essential nutrients causes a transition of yeast cells to a state where they are not only more resistant to nutrient starvation, but also to a severe heat shock (Pringle and Hartwell, 1981). It was demonstrated later that not only mild heat stress, but e.g. also incubation of yeast to high ethanol concentrations, sorbate and low external pH confer heat resistance when cells are subsequently heated following the removal of the stress agent (Plesset *et al.*, 1982; Coote *et al.*, 1991, Cheng and Piper, 1994). Cells exposed to mild osmotic stress were not only shown to become osmotolerant but also exhibited increased thermotolerance (Trollmo *et al.*, 1988; Varela *et al.*, 1992). While all these and some other observations are consistent with a central role of STREs in the induction of stress resistance, no cross-protection by a mild heat shock against osmotic stress had been observed in the latter two studies. This aspect was reinvestigated, and clear evidence for cross-protection against a severe osmotic shock by heat stress was obtained. The cross-protection response observed in yeast is similar in several aspects to that observed in bacteria and may be typical for most or all microbes, which appear to be pessimistic organisms: exposure to one stress induces responses that anticipate exposure to a second or third stress unrelated to the first one. In contrast to standard laboratory growth conditions, exposure to various types of stress is quite common in nature. A control element like STRE, which integrates the effects of different stress signals and triggers an optimal transcriptional output leading to adaptation to unfavourable environmental conditions should therefore provide great selective advantage.

Materials and methods

Yeast strains and media

The *S.cerevisiae* strain GG18 (MATa, *leu2 ura3 his3 trp1 ade8 cta1-2 CTT1-18/7x-LEU2-lacZ*) was obtained by integrative transformation of strain GA74-6A with the pLS9 (Sarokin and Carlson, 1986) derivative pCTT1-18/7x (Marchler *et al.*, 1993). URA$^+$ transformants were tested by Southern analysis and a single copy integrant was selected. Isogenic derivatives of GG18 were constructed. Gene disruptions were also produced in strains W303-1A (MATa, *leu2 ura3 his3 trp1 ade2 can1*; obtained from R.Rothstein, Columbia University, New York) and YPH499 (MATa *leu2 ura3 his3 trp1 ade2 lys2*; obtained from P.Hieter, Johns Hopkins University, Baltimore). Strains were grown in YPD medium (Fink, 1970) at 30°C. In experiments involving various stress conditions, cells from an overnight culture were diluted in YPD to an OD_{600} of 0.1. Cultures were grown to an OD_{600} of 1–1.5 and concentrated solutions of the stress agent were added (pH of medium at time of addition of stress agent: 5.8). For control experiments, solutions were replaced by the same volume of water. Cultures were then incubated at 30°C. After the indicated times the cells were harvested, washed and frozen. When the effect of different mutations in the *HOG1* gene on catalase T induction was tested, strain JBY13 transformed with single copy vector pRS316 (Sikorski and Hieter, 1989) or with the same vector containing wild type or mutant *HOG1* genes were grown on YPD to logarithmic phase before induction with 0.3 M NaCl.

Yeast transformation, plasmids, gene disruptions

Yeast transformation was carried out as described by Ito *et al.* (1983). The following derivatives of GG18 were obtained by gene disruption: GG18hog1::TRP1; as described by Brewster *et al.* (1993). GG18pbs2::LEU2; by using a 3.6 kb *Sac*I–*Spe*I fragment obtained after replacing a 1.2 kb *Nhe*I–*Hin*dIII fragment of *PBS2* (Boguslawski, 1992) by *LEU2*. GG18bcy1::LEU2; using a 6.5 kb *Bam*HI fragment isolated from a plasmid produced starting from a *bcy1::URA3* disruption plasmid (Cannon and Tatchell, 1987) by replacing *URA3* by *LEU2*. GG18*ras2::ADE8*; using a 4.6 kb *Hin*dIII–*Xba*I fragment obtained from a plasmid produced by replacing the *LEU2* gene of plasmid pRa530 (Tatchell *et al.*, 1984) by *ADE8*. Strain W303-1Actt1 was produced by deleting the *CTT1* gene of strain W303-1A using a 2.2 kb *Eco*RI fragment obtained after replacing a *Pst*I–*Sst*I fragment of *CTT1* (Hartig and Ruis, 1986) by *URA3*. pCTT1-18/7x has been described previously (Marchler *et al.*, 1993). JBY13 (MATa *leu2 ura3 his3 trp1 ade2 lys2 hog1::TRP1*) was obtained from a genetic cross between JBY10 (Brewster *et al.*, 1993) and YPH102 (Sikorski and Hieter, 1989). The plasmid pJB30 was constructed by insertion of *HOG1* into the polylinker of the high copy 2μm-based pRS426 plasmid (Sikorski and Hieter, 1989).

Preparation and Northern analysis of RNA

Yeast RNA was isolated as previously described (Richter *et al.*, 1980), subjected to electrophoresis, blotted and hybridized essentially as described by Thomas (1980). Band intensities were evaluated using a PhosphorImager (Molecular Dynamics). The following gene probes were used: a 2.4 kb *Hin*dIII–*Bam*HI *CTT1* fragment isolated from plasmid pBR322-5109 (Spevak *et al.*, 1983); a 1.5 kb *Hin*dIII fragment of *DDR2* isolated from plasmid pBRA2 (McClanahan and McEntee, 1986); a 1.1 *Eco*RI–*Hin*dIII fragment of the *HSP104* gene obtained from plasmid pYS104 (Sanchez and Lindquist, 1990); a 0.58 kb *Eco*RI–*Pst*I fragment of the *GAC1* gene from plasmid pKK14d2 (donated by W.Spevak); a 2.4 kb *Eco*RI fragment of *UBI4* isolated from plasmid pUB200 (donated by A.Bachmair) and a 1.1 kb *Xho*I–*Kpn*I *ACT1* fragment from plasmid pYA301 (Gallwitz and Sures, 1982).

Enzyme activities

β-Galactosidase activity of crude extracts prepared by breakage of yeast cells with glass beads (Rose and Botstein, 1983) was assayed using *o*-nitrophenyl-β-D-galactoside as substrate (Miller, 1972). Catalase activity of extracts was assayed spectrophotometrically at 240 nm (Beers and Sizer, 1952). Protein concentrations were assayed at 280 nm as described by Layne (1957).

Hog1p tyrosine phosphorylation

Tyrosine phosphorylation was assayed essentially as described by Brewster *et al.* (1993). A *HOG1*-overexpressing yeast strain, JBY13 containing

the plasmid pJB30, was used to facilitate detection of Hog1p tyrosine phosphate.

Production of anti-Hog1p antiserum

The Hog1p-coding region was amplified by PCR and inserted between the *Nde*I and *Bam*HI sites of the pET15b bacterial expression vector (Novagen). Induction of protein expression was done essentially as described by the supplier. Insertion of *HOG1* into pET15b allowed for the expression of Hog1p fused to a polyhistidine tag. This fusion protein was purified from bacterial lysates over a nickel affinity column and the polyhistidine tag was removed by cleavage at the thrombin site between the tag and Hog1p. Purified Hog1p was used to immunize rabbits at Cocalico, Inc. (Reamstown, PA). We also coupled purified Hog1p to CNBr-activated Sepharose (Pharmacia) and used the resultant Hog1p-Sepharose to purify anti-Hog1p antibodies from rabbit serum.

Oligonucleotide mutagenesis of HOG1

The K52, T174 and Y176 residues of *HOG1* were replaced by arginine, alanine and phenylalanine, respectively, using an oligonucleotide-directed *in vitro* mutagenesis system (Amersham). The entire open reading frame of each mutant gene was sequenced. In each case there were no mutations other than that introduced. The *hog1* mutant genes were each inserted into the low copy plasmid pRS316 (Sikorski and Hieter, 1989) for transformation into yeast.

Effect of severe osmotic stress

Cultures of strains W303-1A and W303-1Actt1 were grown on YPD overnight, diluted with YPD to an OD_{600} of 0.1 and grown to an OD_{600} of 1. Aliquots of cultures were treated for 60 min with 0.3 M NaCl or were incubated for 60 min at 37°C. They were then diluted in fresh YPD medium to an OD_{600} of 0.1 and the NaCl concentration was raised to 3 M by adding concentrated NaCl solution. The cells were then incubated with vigorous shaking for 8 h at 30°C. Viable cells remaining were assayed by plating aliquots on YPD followed by an incubation at 30°C for 48 h.

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